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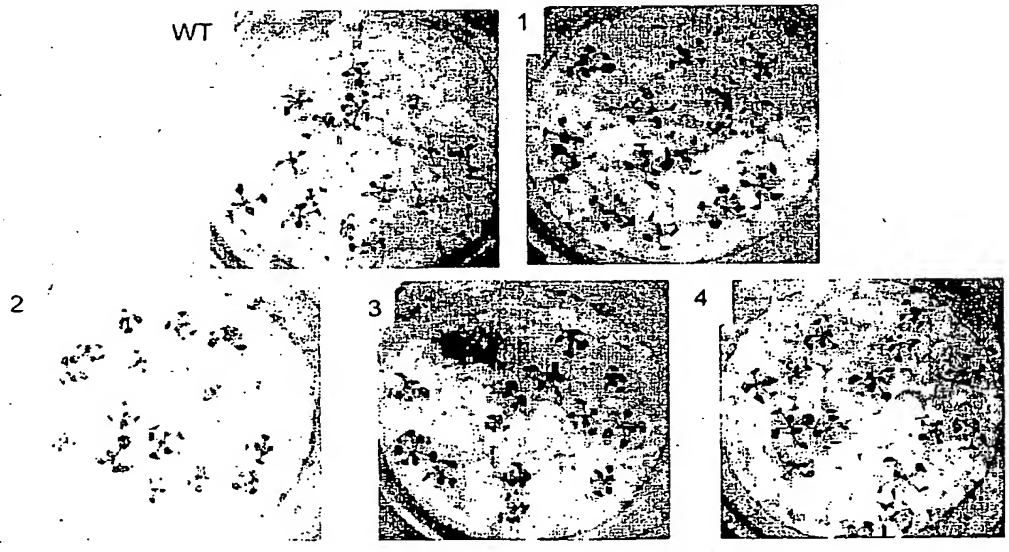
(54) Title: GENETIC MODIFICATION OF PLANTS FOR ENHANCED RESISTANCE AND DECREASED UPTAKE OF HEAVY METALS



(57) Abstract: The present invention relates to a method of producing transformants with enhanced resistance and decreased uptake of heavy metals, and a plant transformed with a P type ATPase ZntA gene that pumps out heavy metals from the cells. The transformants show better growth than wild type in environment contaminated with heavy metals and have lower heavy metal contents than wild type plants. Therefore, this method of transforming plants with ZntA or biologically active ZntA-like heavy metal pumping ATPases can be useful for developing plants for phytoremediation and also for a safe crop that has resistance to heavy metals and low heavy metal contents.

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GENETIC MODIFICATION OF PLANTS FOR ENHANCED RESISTANCE AND DECREASED UPTAKE OF HEAVY METALS

BACKGROUND OF THE INVENTION

(a) Field of the Invention

The present invention relates to a method of producing transformants with enhanced heavy metal resistance. More particularly, the present invention relates to transgenic plants that have an improved growth but decreased heavy metal contents when grown in environment contaminated with heavy metals, thus this method can be used for developing plants for phytoremediation and also for developing safe crops.

(b) Description of the Related Art

Heavy metals are major environmental toxicants, which cause reactive oxidation species generation, DNA damage, and enzyme inactivation by binding to active sites of enzymes in cells.

Contamination of the environment with heavy metals has increased drastically due to industrialization. By the early 1990s, the worldwide annual release had reached 22,000 tons of cadmium, 954,000 tons of copper, 796,000 tons of lead, and 1,372,000 tons of zinc (Alloway BJ & Ayres DC (1993) Principles of environmental pollution. Chapman and Hall, London). The soils contaminated with heavy metal inhibit normal plant growth and cause contamination of foodstuffs. Many heavy metals are very toxic to human health and carcinogenic at low concentrations. Therefore removal of

heavy metals from the environment is an urgent issue.

Studies for removing heavy metals from soil are very actively progressing worldwide. Traditional methods of dealing with soil contaminants include physical and chemical approaches, such as the removal and burial of the contaminated soil, isolation of the contaminated area, fixation (chemical processing of the soil to immobilize the metals), and leaching using an acid or alkali solution (Salt DE, Blaylock M, Kumar NPBA, Viatcheslav D, Ensley BD, et al. (1995). Phytoremediation: a novel strategy for the removal of toxic metals from the environment using plants. *Bio-Technology* 13,468-74; Raskin I, Smith RD, Salt DE. (1997) Phytoremediation of metals: using plants to remove pollutants from the environment. *Curr. Opin. Biotechnol.* 8, 221-6). These methods, however, are costly and energy-intensive processes.

Phytoremediation has recently been proposed as a low-cost, environment-friendly way to remove heavy metals from contaminated soils, and is a relatively new technology for cleanup of contaminated soil that uses general plants, specially bred plants, or transgenic plants to accumulate, remove, or detoxify environmental contaminants. The phytoremediation technology is divided into phytoextraction, rhizofiltration, and phytostabilization.

Phytoextraction is a method using metal-accumulating plants to extract metals from soil into the harvestable parts of the plants; rhizofiltration is a method using plant roots to remove contaminants from polluted aqueous streams; and phytostabilization is the stabilization of contaminants such as toxic metals in soils to prevent their entry into ground water, also with plants

(Salt et al., *Biotechnology* 13(5): 468-474, 1995).

Examples of phytoremediation are methods using the plants of *Larrea tridentate* species that are particularly directed at the decontamination of soils containing copper, nickel, and cadmium (US Patent No. 5,927,005), and a method using *Brassicaceae* family (Baker et al., *New Phytol.* 127:61-68, 1994).

In addition, phytoremediation using transgenic plants that are generated by introducing genes having resistant activity for heavy metals have been attempted. Examples of heavy metal resistant genes are CAX2 (Calcium exchanger 2), cytochrome P450 2E1, NtCBP4 (Nicotiana tabacum calmodulin-binding protein), GSHII (glutathione synthetase), merB (organomercurial lyase), and MRT polypeptide (metal-regulated transporter polypeptide).

CAX2 (Calcium exchanger 2), isolated from *Arabidopsis thaliana*, accumulates heavy metals including cadmium and manganese in plants (Hirschi et al., *Plant Physiol.* 124:125-134, 2000). Cytochrome P450 2E1 uptakes and decomposes organic compounds such as trichloroethylene (Doty SL et al., *Proc. Natl. Acad. Sci.* USA 97:6287-6291, 2000). *Nicotiana tabacum* transformed with NtCBP4 has resistant activity for nickel (Arazi et al., *Plant J.* 20:171-182, 1999), GSHII accumulates cadmium (Liang et al., *Plant Physiol.* 119:73-80, 1999), merB detoxifies organic mercury (Bizily et al., *Proc. Natl. Acad. Sci.* USA 96:6808-6813, 1999), and MRT polypeptide removes heavy metals including cadmium, zinc, and manganese from contaminated soil (US Patent No. 5,846,821).

However, the transgenic plants generated by introducing the above-mentioned genes have limitations in growth due to accumulation of heavy metals, and they can produce contaminated fruits and crops, when grown in contaminated soil. Therefore, there is a need for plants that have a lower uptake of heavy metals than the wild type, and that maintain healthy growth even in an environment contaminated with heavy metals.

SUMMARY OF THE INVENTION

It is an object of the invention to provide a gene, when expressed in plants, that confers heavy metal resistance and that can inhibit accumulation of heavy metals.

It is a further object of the invention to provide a recombinant vector harboring a heavy metal resistant gene.

It is a further object of the invention to provide a method for producing transformants that have heavy metal resistance and that accumulate less heavy metals than wild type plants.

It is a further object of the invention to provide transformants that have heavy metal resistance and that accumulate less heavy metals than wild type plants.

It is a further object of the invention to provide a method of transforming a polluted area into an environmentally friendly space.

To accomplish the aforementioned objects, the invention provides a recombinant vector containing a coding sequence for a heavy metal-transporting P type ATPase, wherein the coding sequence is operably

linked to and under the regulatory control of a plant-expressible transcription and translation regulatory sequence.

Also, the invention provides a transgenic plant, or parts thereof, each transformed with a recombinant vector.

Also, the invention provides a transgenic plant cell.

Also, the invention provides a transgenic plant, stably transformed with a recombinant vector.

Also, the invention provides a recombinant vector comprising a coding sequence for a heavy metal-transporting P type ATPase, ZntA of SEQ ID NO: 1;

wherein the coding sequence is operably linked to and under the regulatory control of a plant-expressible transcription and translation regulatory sequence; and

wherein the ZntA contains an approximately 100 amino acid residue N-terminal extension domain, a first transmembrane spanning domain, a second transmembrane spanning domain containing a putative cation channel motif CPX domain, a third transmembrane spanning domain, a first cytoplasmic domain, a second cytoplasmic domain, and a C-terminal domain

Also, the invention provides a recombinant vector comprising a coding sequence for a heavy metal-transporting P type ATPase, ZntA wherein the coding sequence is operably linked to and under the regulatory control of a plant-expressible transcription and translation regulatory;

wherein the ZntA contains an approximately 100 amino acid residue N-terminal extension domain, a first transmembrane spanning domain, a

second transmembrane spanning domain containing a putative cation channel motif CPX domain, a third transmembrane spanning domain, a first cytoplasmic domain, a second cytoplasmic domain, and a C-terminal domain; and

wherein each of the domains of the coding sequence shares at least about 50% homology with a same domain of SEQ ID NO:1.

Also, the invention provides a method of producing a transgenic plant with enhanced resistance to heavy metals comprising:

(a) preparing an expression construct comprising a sequence encoding a heavy metal-transporting P type ATPase, operably linked to and under the regulatory control of a plant-expressible transcription and translation regulatory sequence;

(b) preparing a recombinant vector harboring the expression construct; and

(c) introducing the expression construct of the recombinant vector into a plant cell or plant tissue to produce a transgenic plant cell or transgenic plant tissue

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 represents the map of the recombinant vector pEZG.

Fig. 2 shows plasma membrane localization of ZntA protein expressed in Arabidopsis protoplasts.

Fig. 3 is a Western blot photograph showing membrane localization of ZntA protein expressed in Arabidopsis protoplast.

Fig. 4 represents the map of recombinant vector PBI121/*zntA*.

Fig. 5 is a Northern blot photograph showing expression of *zntA* mRNA in *Arabidopsis*.

Fig. 6 shows the enhanced growth of *zntA*-transgenic plants over that of wild type in a medium containing lead.

Fig. 7 shows the enhanced growth of *zntA*-transgenic plants over that of wild type in a medium containing cadmium.

Fig. 8 is a graph showing the weight of *zntA*-transgenic pants cultivated in media containing heavy metals.

Fig. 9 is a graph showing the chlorophyll contents of *zntA*-transgenic and wild type plants, grown in media containing heavy metals.

Fig. 10 is a graph showing the heavy metal contents of *zntA*-transgenic and wild type plants, grown in media containing heavy metals.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

As used herein, the term "P type ATPase"" refers to a transporter that transports a specific material by using energy from ATP hydrolysis and that forms a phosphorylated intermediate. More particularly, the P type ATPase is a heavy metal-transporting ATPase. The heavy metal is a metal element having a specific gravity over 4 including arsenic(As), antimony(Sb), lead(Pb), mercury(Hg), cadmium(Cd), chrome, tin(Sn), zinc, barium(Ba), nickel(Ni), bismuth(Bi), cobalt(Co), manganese(Mn), iron(Fe), copper(Cu), and vanadium(V).

ZntA is a P type ATPase of *E.coli* (Rensing C, Mitra B, Rosen BP. (1997) *Proc. Natl. Acad. Sci.* U S A. 94,14326-31; Sharma, R., Rensing, C., Rosen, B. P., Mitra, B. (2000) *J Biol Chem.* 275,3873-8) which pumps Pb(II)/Cd(II)/Zn(II) across the plasma membrane.

P-type ATPases typically have 2 large cytoplasmic domains and 6 transmembrane domains. ZntA has similar domains, and in addition, 2 more transmembrane helixes at N-terminus and N-terminal extension of about 100 amino acids containing CXXC motif. The first large cytoplasmic domain of ZntA is about 145 amino acid long and involved in hydrolysis of phosphointermediate, and the second large cytoplasmic domain is 280 amino acid long and has a phosphorylation motif. We denote the 4 transmembrane helixes of the N-terminal side as the first transmembrane spanning domain. The 2 transmembrane helixes between the 2 large cytoplasmic domains is denoted as the second transmembrane spanning domain. This domain contains a putative cation channel motif CPX domain. The transmembrane helixes between the second large cytoplasmic domain and the c-terminus is denoted as the third transmembrane spanning domain. The cytoplasmic domain following the third transmembrane spanning domain is denoted as the C-terminal domain of ZntA.

The term "homology" refers to the sequence similarity between 2 DNA or protein molecules. "Biologically active ZntA-like heavy metal pumping ATPases" are coded by DNA sequences which have at least 50% homology to ZntA, and have heavy metal pumping activity. Biologically active ZntA-like heavy metal pumping ATPases include zinc-transporting

ATPase (NC_000913), zinc-transporting ATPase (NC_002655), heavy metal-transporting ATPase (NC_003198), P-type ATPase family (NC_003197), cation transporting P-type ATPase from *Mycobacterium lepraed* (GenBank #Z46257), and many others.

A "heavy metal resistance protein" is a protein capable of mediating resistance to at least one heavy metal, including, but not limited to, lead, cadmium, and zinc. An example of a heavy metal resistance protein is ZntA protein of SEQ ID NO:1.

The term "plant-expressible" means that the coding sequence is operably linked to and under the regulatory control of a transcription and translation regulatory sequence that can be efficiently expressed by plant cells, tissues, parts and whole plants.

"Plant-expressible transcriptional and translational regulatory sequences" are those which can function in plants, plant tissues, plant parts and plant cells to effect the transcriptional and translational expression of the target sequence with which they are associated. Included are 5' sequences of a target sequence to be expressed, which qualitatively control gene expression (turn gene expression on or off in response to environmental signals such as light, or in a tissue-specific manner); and quantitative regulatory sequences which advantageously increase the level of downstream gene expression. An example of a sequence motif that serves as a translational control sequence is that of the ribosome binding site sequence. Polyadenylation signals are examples of transcription regulatory sequences positioned downstream of a target sequence, and there are

several that are well known in the art of plant molecular biology.

A "transgenic plant" is one that has been genetically modified, unlike the wild type plants. Transgenic plants typically express heterologous DNA sequences, which confer the plants with characters different from that of wild type plants. As specifically exemplified herein, a transgenic plant is genetically modified to contain and express at least one heterologous DNA sequence that is operably linked to and under the regulatory control of transcriptional control sequences which function in plant cells or tissue, or in whole plants.

The present invention provides a plant-expressible expression construct containing a coding sequence for a heavy metal-transporting ATPase protein. The coding sequence is operably linked to and under the regulatory control of a plant-expressible transcription and translation regulatory sequence. The heavy metals include arsenic(As), antimony(Sb), lead(Pb), mercury(Hg), cadmium(Cd), chrome, tin(Sn), zinc, barium(Ba), nickel(Ni), bismuth(Bi), cobalt(Co), manganese(Mn), iron(Fe), copper(Cu) and vanadium(V).

The expression construct includes a promoter, a heavy metal-transporting P type ATPase gene, and a transcriptional terminator. The suitable plant-expressible promoters include the 35S or 19S promoters of Cauliflower Mosaic Virus; the nos (nopaline synthase), ocs (octopine synthase), or mas (mannopine synthase) promoters of *Agrobacterium tumefaciens* Ti plasmids; and others known to the art.

The heavy metal-transporting ATPase gene of the present invention

prefers genes encoding ZntA (SEQ ID NO:1) or biologically active ZntA-like heavy metal pumping ATPase genes, which have at least 50% homology to ZntA, and which code for proteins with heavy metal pumping activities.

The heavy metal-transporting ATPase gene of the present invention also prefers DNA sequences containing an approximately 100 amino acid residue N-terminal extension domain, a first transmembrane spanning domain, a second transmembrane spanning domain containing a putative cation channel motif CPX domain, a third transmembrane spanning domain, a first cytoplasmic domain, a second cytoplasmic domain, and a C-terminal domain of ZntA, or DNA sequences which share at least 50% homology with abovementioned domains of the biologically active ZntA-like heavy metal pumping ATPase genes.

The expression construct of the present invention may further contain a marker allowing selection of transformants in the plant cell or showing a localization of a target protein. The examples of a marker are genes carrying resistance to an antibiotic such as kanamycin, hygromycin, gentamicin, and bleomycin; and genes coding *GUS* (β -glucuronidase), *CAT* (chloramphenicol acetyltransferase), luciferase, and *GFP* (green fluorescent protein). The marker allows for selection of successfully transformed plant cells growing in a medium containing certain antibiotics because they will carry the expression construct with the resistance gene to the antibiotic.

Also, the invention provides a recombinant vector comprising the expression construct. The recombinant vector comprises a backbone of the common vector and the expression construct. The common vector is

preferably selected from the group consisting of pROKII, pBI76, pET21, pSK(+), pLSAGPT, pBI121, and pGEM. Examples of the prepared recombinant vector are PBI121/*zntA* and pEZG. PBI121/*zntA* comprises a backbone of PBI121, CMV 35S promoter, *zntA* gene, and nopaline synthase terminator; and pEZG comprises a backbone of pUC, CMV 35S promoter, *zntA* gene, green fluorescence protein, and nopaline synthase terminator.

Also, the present invention provides a transformant containing the expression construct. The transformant contains a DNA sequence encoding a heavy metal-transporting P type ATPase, wherein the coding sequence is operably linked to and under the regulatory control of a transcription and translation regulatory sequence.

The transformant is preferably a plant, and more preferably a plant, parts thereof, and plant cell. The plant parts include a seed. The plants are herbaceous plants and trees, and they include flowering plants, garden plants, an onion, a carrot, a cucumber, an olive tree, a sweet potato, a potato, a cabbage, a radish, lettuce, broccoli, *Nicotiana tabacum*, *Petunia hybrida*, a sunflower, *Brassica juncea*, turf, Arabidopsis thaliana, *Brassica campestris*, *Betula platyphylla*, a poplar, a hybrid poplar, and *Betula schmidtii*.

Techniques for generating transformants are well known. An example is *Agrobacterium tumefaciens*-mediated DNA transfer. Preferably, recombinant *A. tumefaciens* generated by electroporation, micro-particle injection, or with a gene gun can be used.

In addition, the invention provides a method of producing a transgenic plant with enhanced resistance to heavy metals, comprising:

(a) preparing an expression construct comprising a plant-expressible sequence encoding a heavy metal-transporting P type ATPase, operably linked to and under the regulatory control of a transcription and translation regulatory sequence;

(b) preparing a recombinant vector harboring the expression construct; and

(c) introducing the expression construct of the recombinant vector into a plant cell or plant tissue to produce a transgenic plant cell or transgenic plant tissue.

The method of producing a transgenic plant further comprises a step: (d) regenerating a transgenic plant from the transgenic plant cell or transgenic plant tissue of step (c).

In the present invention, ZntA protein was expressed in the plasma membrane (Figs. 2 and 3). Moreover, *zntA*-transgenic *Arabidopsis* plants showed enhanced resistance to lead and cadmium, and the content of lead and cadmium was lower than in a wild-type plant.

Therefore, *zntA*-transgenic plants or plants transformed with a gene encoding biologically active ZntA-like heavy metal pumping ATPases can grow in an environment contaminated with heavy metals, and this technique can be useful for generating crop plants with decreased uptake of harmful heavy metals. Since harmful heavy metals can be introduced into farmland inadvertently, for example, due to the yellow sand phenomenon or by natural disaster, heavy metal pumping transgenic crop plants can be a safe choice for health-concerned consumers.

The following examples are provided for illustrative purposes and are not intended to limit the scope of the invention as claimed herein. Any variations in the exemplified compositions and methods which occur to the skilled artisan are intended to fall within the scope of the present invention.

EXAMPLE 1.

Isolation of *zntA* gene

Escherichia coli K-12 was obtained from the Korean Collection for Type Cultures of the Korea Research Institute of Bioscience and Biotechnology, and a zntA gene was cloned.

zntA was isolated by PCR using genomic DNA of *Escherichia coli* K-12 strain as a template. PCR was performed with a primer set of SEQ ID NO:2, SEQ ID NO:3, and 2.2 kb of PCR product, and *zntA* of SEQ ID NO:1 was obtained. The sequence of the PCR product was analyzed and the PCR product was cloned into a pGEM-T easy vector to produce pGEM-T/*zntA*.

EXAMPLE 2.

Expression of ZntA protein

A *zntA* gene was introduced into *Arabidopsis* protoplasts, and localization of ZntA protein was investigated.

(2-1) Preparation of *Arabidopsis* protoplasts

Arabidopsis protoplasts were prepared as described (Abel S, Theologis A (1994) Transient transformation *of Arabidopsis* leaf protoplasts: a versatile experimental system to study gene expression. *Plant J.* 5, 421-7).

Seeds of *Arabidopsis* were placed into an antiseptic solution (distilled water: chlorox: 0.05% triton X-100 = 3:2:2), shaken for 20-30 seconds, and incubated at room temperature for 5-10 mins. The seeds were then rinsed five times with distilled water.

The *Arabidopsis* seeds were incubated in 100 ml of a liquid solution (Murashige & Skoog medium; MSMO, pH 5.7-5.8) containing vitamins, Duchefa 4.4 g/L, sucrose 20 g/L, MES (2-(N-Morpholino) Ethanesulfonic acid, Sigma) 0.5 g/L, while agitating at 120 rpm under a 16/8 hr (light/dark) cycle, at 22 ℃ for 2-3 weeks.

The 2-3 week-old whole plants were chopped with a razor blade to 5-10 mm^2 pieces. These leaf fragments were transferred to an enzyme solution (1% cellulase R-10, 0.25% marcerozyme R-10, 0.5 M mannitol, 10 mM MES, 1 mM $CaCl_2$, 5 mM β -mercaptoethanol, and 0.1% BSA, pH 5.7-5.8), vacuum-infiltrated for 10 min, and then incubated in the dark at 22 ℃ for 5 hours with gentle agitation at 50-75 rpm. The released protoplasts were filtered through a 100 ㎛ mesh (Sigma S0770, USA), purified using a 21% sucrose gradient by centrifugation at 730 rpm for 10 min, and then suspended in 20 ml of W5 solution (154 mm NaCl, 125 mM $CaCl_2$, 5 mM KCl, 5 mM glucose, and 1.5 mM MES, pH 5.6) and centrifuged again at 530 rpm for 6 min. The pellected protoplasts were re-suspended in W5 solution and kept on ice.

(2-2) Preparation of vector

pGEM-T/*zntA* DNA was cut with *BamHI* restriction enzyme and *zntA*

genes were extracted (QIAGEN Gel extraction kit). The *zntA* genes were placed under the control of a Cauliflower Mosaic Virus 35S promoter, fused with and then inserted into a pUC-GFP vector containing Green Fluorescent Protein(GFP) and nopaline synthase terminator(NOS), to thereby produce pEZG.

(2-3) Preparation of vector for H^+ pumping gene

A hydrogen ion pump gene of *Arabidopsis*, AHA2 cDNA (Gene Bank: P19456), was amplified by PCR. Primers for PCR were polynucleotides of SEQ ID NO:4 and SEQ ID NO:5. PCR conditions were as follows: 94 ℃, 30 sec -> 45 ℃, 30 sec -> 72 ℃, 1 min, 50 cycles. The PCR product was obtained as AHA2 cDNA.

A DsRed vector (Clontech, Inc.) was treated with *Bg*III/*Not*I restriction enzyme and DsRed was obtained. The DsRed was inserted into the opened smGFP vector with a *BamH*I/*Ecl136*II restriction enzyme to 326RFP. In addition, AHA2 cDNA was inserted at *Xma*I of the 326RFP vector and 326RFP/AHA2 was prepared.

(2-4) Introduction of pEZG or 326RFP/AHA2 into protoplast

pEZG and 326RFP/AHA2 were introduced to the protoplasts prepared by EXAMPLE (2-1), and expression of foreign genes was confirmed.

The protoplast was centrifuged at 500 rpm for 5 min, and 5 X 10^6/ml of the protoplast were suspended in a MaMg solution (400 mM mannitol, 15 mM $MgCl_2$, 5 mM MES-KOH, pH 5.6). 300 $\mu\ell$ of the suspension solution

was mixed with 10 μg of pEZG and 326RFP/AHA2 respectively, which was then was added to 300 $\mu \ell$ of PEG (400 mM mannitol, 100 mM $Ca(NO_3)_2$, 40% PEG 6000), and stored at RT for 30 min. The mixture was washed with 5 ml of W5 solution, centrifuged at 500 rpm for 3 min, and a pellet was obtained. The pellet was washed with 2 ml of W5 solution and incubated in the dark at 22-25 ℃. After 24 hr, expression of GFP protein was monitored and images were captured with a cooled charge-coupled device camera using a Zeiss Axioplan fluorescence microscope. The filter sets used for the GFP were XF116 (exciter, 474AF20; dichroic, 500DRLP; emitter, 510AF23) (Omega, Inc., Brattleboro, VT). Data were then processed using Adobe (Mountain View, CA) Photoshop software.

Fig. 2 shows a localization of ZntA protein fused with GFP in protoplasts transformed with pEZG and 326RFP/AHA2, respectively. "a" is control, "b" is AHA2 protein expressed in 326RFP/AHA2, "c" is ZntA protein expressed in pEZG, and "d" is an overlapped picture of "b" and "c". ZntA fused with GFP shows a green color due to GFP, and AHA2 fused with DsRed shows a red color due to DsRed.

In Fig. 2, ZntA fused with GFP was localized at the plasma membrane in *Arabidopsis* protoplasts.

In addition, membrane and cytosol fractions were isolated from *Arabidopsis* protoplasts, and Western Blot was preformed using a GFP antibody as a probe. Fig. 3 is a Western Blot photograph, wherein "WT-C" is cytosol of wild-type *Arabidopsis* protoplasts, "WT-M" is membrane of

wild-type *Arabidopsis* protoplasts, "ZntA-C" is cytosol of *Arabidopsis* protoplasts transformed with pEZG, and " ZntA-M" is membrane of *Arabidopsis* protoplasts transformed with pEZG. In Fig. 3, the GFP antibody cross-reacted only with membrane proteins extracted from *Arabidopsis* protoplasts transformed with pEZG, confirming that ZntA protein was expressed in membrane.

EXAMPLE 3.

Preparation of transgenic plants expressing ZntA protein.

(3-1) *Arabidopsis*

Arabidopsis plants were grown at 4 ℃ for 2 days, then they were grown with a 16/8 hr (light/dark) photoperiod, at 22 ℃/18 ℃ for 3-4 weeks until flower stalks were differentiated. The 1st flower stalk was removed, and the 2nd flower stalk was used for transformation.

(3-2) pBI121/ *zntA* vector

A *zntA* gene was inserted into the expression vector for the plant, preparing pBI121 and pBI121/zntA.

A GUS gene of pBI121 was removed by digesting with *Sma*I and *Ecl136*II restriction enzymes, and a *zntA* gene prepared from the pGEM-T/*zntA* was inserted to pBI121, thereby preparing a pBI121/*zntA* vector (Fig. 4).

(3-3) Preparation of transgenic plants

pBI121/*zntA* vector DNA was isolated with a prep-kit (Qiagen) and introduced to agrobacterium using electroporation. The agrobacterium

(KCTC 10270BP) was cultured in YEP media (yeast extract 10 g, NaCl 5 g, pepton 10 g, pH 7.5) until index of O.D. reached 0.8-1.0. The culture solution was centrifuged, cells were collected and suspended in MS media (Murashige & Skoog medium, 4.3 g/L, Duchefa) containing 5% sucrose, and Silwet L-77 (LEHLE SEEDS, USA) was added as a final concentration of 0.01% just before transformation. For plant transformation, pBI121/*zntA* was introduced into the *Agrobacterium* LBA4404 strain, which was then used to transform *Arabidopsis* by a dipping method (Clough SJ, and Bent AF (1988), Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J*. 16, 735-743).

EXAMPLE 4.

Selection of transformants

For selection of plant transformed with *zntA* genes, plants were grown in solid Murashige-Skoog (MS) medium containing kanamycin (50 mg/l). T2 or T3 generation seeds were used for the tests. Also, a pBI121 vector was introduced to *Arabidopsis* and transformants (pBI121 plants) were selected. Seeds were obtained from wild-type *Arabidopsis*, pBI121 plants, and pBI121/*zntA* plants, respectively.

To test the ZntA expression level, total RNA was isolated from kanamycin-selected T2 plants and used for Northern Blot analysis. Total RNA was extracted from *Arabidopsis* plants grown on the 1/2 MS (Murashige & Skoog medium, 2.15 g/L, Duchefa)-agar media for 3 weeks. Subsequent RNA preparation and northern hybridization followed the established method (Sambrook et al. (2001) *Molecular Cloning: A laboratory manual* (Third

Edition), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY) with slight modifications.

The plant materials were frozen in liquid nitrogen and homogenized with mortars and pestles. 1 ml of TRIzol reagent (Life technology, USA) per 100 mg of tissue was added to the sample and after 5 min incubation at RT, 0.2 ml of chloroform per 1 ml of TRIzol reagent was added. After centrifugation at 10,000g for 10 min at 4°C, the aqueous phase was taken and precipitated with 0.5 ml of isopropyl alcohol per 1 ml of TRIzol reagent and quantified by UV spectroscopy. Total RNA was separated in a formaldehyde-containing agarose gel and then transferred onto a nylon membrane. After UV crosslinking, hybridization was carried out in a modified Church buffer (7% (w/v) SDS, 0.5 M sodium phosphate (pH 7.2), 1 mM EDTA (pH 7.0)) at 68°C overnight, with ^{32}P-labeled *zntA* probes. Membranes were washed once for 10 min in 1 x SSC, 0.1% SDS at room temperature, and twice for 10 min in 0.5 x SSC, 0.1% SDS at 68°C. The membrane was exposed to a phosphorimager screen (Fuji film) or x-ray film (Kodak). The mRNA expression levels were analyzed by the Mac-BAS image-reader program. Fig. 5 is a Northern Blot photograph showing expression of *zntA* mRNA in *Arabidopsis*. Transcription of *zntA* RNA was not observed in wild-type *Arabidopsis* and pBI121 plants, but it was observed in pBI121/*zntA* plants. EF1-a is constitutively expressed in plants and its even levels indicated that the same amount of RNA was used for different samples.

EXAMPLE 5.

Heavy metals resistance of plant transformed with *zntA* gene

Wild-type *Arabidopsis* plants and pBI121/*zntA* plants were grown in 1/2 MS-agar media for 2 weeks and transferred 1/2 MS-liquid media containing 70 μ M cadmium or 0.7 mM lead. After 2 weeks, growth, weight, and heavy metal contents were measured.

(5-1) Growth of plants

Fig. 6 shows the growth of wild-type and pBI121/*zntA Arabidopsis* plants grown in a medium containing lead. Fig. 7 shows wild-type and pBI121/*zntA Arabidopsis* plants grown in a medium containing cadmium. "WT" is wild-type *Arabidopsis*, "1" to "4" are pBI121/*zntA* plants. In Figs. 6 and 7, pBI121/*zntA* plants grew better than the wild-type plants; their leaves were broader, greener, and their fresh weights were higher than those of the wild types. These results indicate that the expression of ZntA confers Pb(II)- and Cd(II)-resistance to the transgenic plants.

(5-2) Measurement of biomass

Wild type and pBI121/*zntA Arabidopsis* plants were grown in 1/2 MS-agar media for 2 weeks and then transferred to 1/2 MS-liquid media supported by small gravel with or without Cd (II) or Pb (II). After growing for an additional 2 weeks, the plants were harvested. They were washed in an ice-cold 1 mM tartarate solution and blot-dried. The weight of the *wild type* and pBI121/*zntA Arabidopsis* plants were measured.

Fig. 8a is a graph showing the weight of wild type and pBI121/*zntA* plants grown in a medium containing lead, and Fig 8b is a graph showing the weight of wild type and pBI121/*zntA* plants grown in a medium containing

cadmium. The weight of pBI121/*zntA* plants was higher than that of the wild-type plants. These results indicate that plants expressing ZntA protein can grow better than wild type in soil contaminated with heavy metals.

(5-3) Measurement of chlorophyll contents

For determination of chlorophyll contents, the leaves were harvested and extracted with 95% ethanol for 20 min at 80 ℃. Absorbance at 664 nm and 648 nm were measured and then the chlorophyll A and B contents were calculated as described (Oh SA, Park JH, Lee GI, Paek KH, Park SK, Nam HG (1997) Identification of three genetic loci controlling leaf senescence in Arabidopsis thaliana. Plant J. 12, 527-35).

Fig. 9a is a graph showing the chlorophyll contents of wild type and *zntA*-transgenic plants grown in a medium containing lead, and Fig. 9b is a graph showing the chlorophyll contents of wild type and *zntA*-transgenic plants grown in a medium containing cadmium. The chlorophyll contents of *zntA*-transgenic plants were higher than those of the wild types.

(5-4) Measurement of the heavy metal contents

We measured the content of Pb and Cd in control and ZntA overexpressing plants grown in media containing heavy metals. pBI121/*zntA* plants were collected, weighed, and digested with 65% HNO_3 at 200°C, overnight. Digested samples were diluted with 0.5 N HNO_3 and analyzed using an atomic absorption spectrometer (AAS; SpectrAA-800, Varian).

Fig. 10 is a graph showing the heavy metal contents of wild type and *zntA*-transgenic plants grown in media containing heavy metals. Fig. 10a is the lead contents, and 10b is the cadmium contents. Pb content of

pBI121/*zntA* plants varied between the lines, but it was consistently lower than that of the wild type. Cd content in transgenic lines 1 and 3 was lower than that in the control.

Thus, plants transformed with *zntA* or other biologically active ZntA-like heavy metal pumping ATPases can be grown in soil contaminated with heavy metals and have less uptake of heavy metals than wild type plants. Since growing plants can hold contaminated soil and thereby reduce erosion of the soil, and since the zntA-transgenic plants can grow better than wild type plants in soil contaminated by heavy metals, they can reduce migration of pollutants from the polluted area, thereby reducing contamination of groundwater by the pollutants. The present invention can also be applied to crop plants to produce low heavy metal –containing safe crop plants.

Applicant's or agent's file reference	International application No.
OPP020276KR	

INDICATIONS RELATING TO DEPOSITED MICROORGANISM OR OTHER BIOLOGICAL MATERIAL

(PCT Rule 13*bis*)

A. The indications made below relate to the deposited microorganism or other biological material referred to in the description on page 19, line 1.

B. IDENTIFICATION OF DEPOSIT — Further deposits are identified on an additional sheet ☐

Name of depositary institution

Korean Collection for Type Cultures

Address of depositary institution *(including postal code and country)*

#52, Oun-dong, Yusong-ku, Taejon 305-333,
Republic of Korea

Date of deposit	Accession Number
March 29, 2002	KCTC 10207BP

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* — This information is continued on an additional sheet ☐

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g., "Accession Number of Deposit")*

For receiving Office use only	For International Bureau use only
☐ This sheet was received with the international application	☐ This sheet was received by the International Bureau on:
Authorized officer	Authorized officer

Form PCT/RO/134 (July 1998)

WHAT IS CLAIMED IS:

1. A recombinant vector comprising a coding sequence for a heavy metal-transporting P type ATPase, wherein the coding sequence is operably linked to and under the regulatory control of a plant-expressible transcription and translation regulatory sequence.

2. The recombinant vector according to Claim 1, wherein the heavy metal is at least one selected from the group consisting of arsenic, antimony, lead, mercury, cadmium, chrome, tin, zinc, barium, nickel, bismuth, cobalt, manganese, iron, copper, vanadium.

3. The recombinant vector according to Claim 1, wherein the P type ATPase is ZntA.

4. The recombinant vector according to Claim 3, wherein the ZntA has an amino acid sequence as given in SEQ ID NO:2.

5. The recombinant vector according to Claim 1, wherein the coding sequence is ZntA-like heavy metal pumping ATPase gene comprising a nucleic acid sequence sharing at least about 50% homology with ZntA as given in SEQ ID NO: 1.

6. The recombinant vector according to Claim 1, wherein the recombinant vector is PBI121/*zntA* or pEZG.

7. A transgenic plant, or parts thereof, each transformed with a recombinant vector of claim 1.

8. The transgenic plant, or thereof according to Claim 7, wherein the heavy metal is at least one selected from the group consisting of arsenic, antimony, lead, mercury, cadmium, chrome, tin, zinc, barium, nickel, bismuth,

cobalt, manganese, iron, copper, vanadium.

9. A transgenic plant cell, transformed with a recombinant vector of claim 1.

10. The transgenic plant cell according to Claims 9, wherein the heavy metal is at least one selected from the group consisting of arsenic, antimony, lead, mercury, cadmium, chrome, tin, zinc, barium, nickel, bismuth, cobalt, manganese, iron, copper, vanadium.

11. A transgenic plant, stably transformed with a recombinant vector of claim 1.

12. The transgenic plant according to Claim 11, wherein the heavy metal is at least one selected from the group consisting of arsenic, antimony, lead, mercury, cadmium, chrome, tin, zinc, barium, nickel, bismuth, cobalt, manganese, iron, copper, vanadium.

13. A transgenic plant, or parts thereof, each transformed with a recombinant vector of claim 5.

14. The transgenic plant, or parts thereof according to Claims 13, wherein the heavy metal is at least one selected from the group consisting of arsenic, antimony, lead, mercury, cadmium, chrome, tin, zinc, barium, nickel, bismuth, cobalt, manganese, iron, copper, vanadium.

15. A transgenic plant cell, transformed with a recombinant vector of claim 5.

16. The transgenic plant cell according to Claims 15, wherein the heavy metal is at least one selected from the group consisting of arsenic, antimony, lead, mercury, cadmium, chrome, tin, zinc, barium, nickel, bismuth,

cobalt, manganese, iron, copper, vanadium.

17. A transgenic plant, stably transformed with a recombinant vector of claim 5.

18. The transgenic plant according to Claim 17, wherein the heavy metal is at least one selected from the group consisting of arsenic, antimony, lead, mercury, cadmium, chrome, tin, zinc, barium, nickel, bismuth, cobalt, manganese, iron, copper, vanadium.

19. A recombinant vector comprising a coding sequence for a heavy metal-transporting P type ATPase, ZntA of SEQ ID NO: 1;

wherein the coding sequence is operably linked to and under the regulatory control of a plant-expressible transcription and translation regulatory sequence; and

wherein the ZntA contains an approximately 100 amino acid residue N-terminal extension domain, a first transmembrane spanning domain, a second transmembrane spanning domain containing a putative cation channel motif CPX domain, a third transmembrane spanning domain, a first cytoplasmic domain, a second cytoplasmic domain, and a C-terminal domain.

20. A transgenic plant, or parts thereof, each transformed with a recombinant vector of claim 19.

21. The transgenic plant, or parts thereof according to Claims 20, wherein the heavy metal is at least one selected from the group consisting of arsenic, antimony, lead, mercury, cadmium, chrome, tin, zinc, barium, nickel, bismuth, cobalt, manganese, iron, copper, vanadium.

22. A transgenic plant cell, transformed with a recombinant vector of

claim 19.

23. The transgenic plant cell according to Claims 22, wherein the heavy metal is at least one selected from the group consisting of arsenic, antimony, lead, mercury, cadmium, chrome, tin, zinc, barium, nickel, bismuth, cobalt, manganese, iron, copper, vanadium.

24. A transgenic plant, stably transformed with a recombinant vector of claim 19.

25. The transgenic plant according to Claim 24, wherein the heavy metal is at least one selected from the group consisting of arsenic, antimony, lead, mercury, cadmium, chrome, tin, zinc, barium, nickel, bismuth, cobalt, manganese, iron, copper, vanadium.

26. A recombinant vector comprising a coding sequence for a heavy metal-transporting P type ATPase, ZntA

wherein the coding sequence is operably linked to and under the regulatory control of a plant-expressible transcription and translation regulatory;

wherein the ZntA contains an approximately 100 amino acid residue N-terminal extension domain, a first transmembrane spanning domain, a second transmembrane spanning domain containing a putative cation channel motif CPX domain, a third transmembrane spanning domain, a first cytoplasmic domain, a second cytoplasmic domain, and a C-terminal domain; and

wherein each of the domains of the coding sequence shares at least about 50% homology with a same domain of SEQ ID NO:1.

27. A transgenic plant, or parts thereof, each transformed with recombinant vector of claim 26.

28. The transgenic plant, or parts thereof according to Claims 27, wherein the heavy metal is at least one selected from the group consisting of arsenic, antimony, lead, mercury, cadmium, chrome, tin, zinc, barium, nickel; bismuth, cobalt, manganese, iron, copper, vanadium.

29. A transgenic plant cell, transformed with a recombinant vector of claim 26.

30. The transgenic plant, or parts thereof according to Claims 29, wherein the heavy metal is at least one selected from the group consisting of arsenic, antimony, lead, mercury, cadmium, chrome, tin, zinc, barium, nickel, bismuth, cobalt, manganese, iron, copper, vanadium.

31. A transgenic plant, stably transformed with a recombinant vector of claim 30.

32. The transgenic plant according to Claim 31, wherein the heavy metal is at least one selected from the group consisting of arsenic, antimony, lead, mercury, cadmium, chrome, tin, zinc, barium, nickel, bismuth, cobalt, manganese, iron, copper, vanadium.

33. A method of producing a transgenic plant with enhanced resistance to heavy metals comprising:

(a) preparing an expression construct comprising a sequence encoding a heavy metal-transporting P type ATPase, operably linked to and under the regulatory control of a plant-expressible transcription and translation regulatory sequence;

(b) preparing a recombinant vector harboring the expression construct; and

(c) introducing the expression construct of the recombinant vector into a plant cell or plant tissue to produce a transgenic plant cell or transgenic plant tissue.

34. The method of producing a transgenic plant according to Claim 33, wherein the heavy metal is at least one selected from the group consisting of arsenic, antimony, lead, mercury, cadmium, chrome, tin, zinc, barium, nickel, bismuth, cobalt, manganese, iron, copper, vanadium.

35. The method of producing a transgenic plant according to Claim 33, further comprising the step of: regenerating a transgenic plant from the transgenic plant cell or transgenic plant tissue of step (c).

Fig. 1

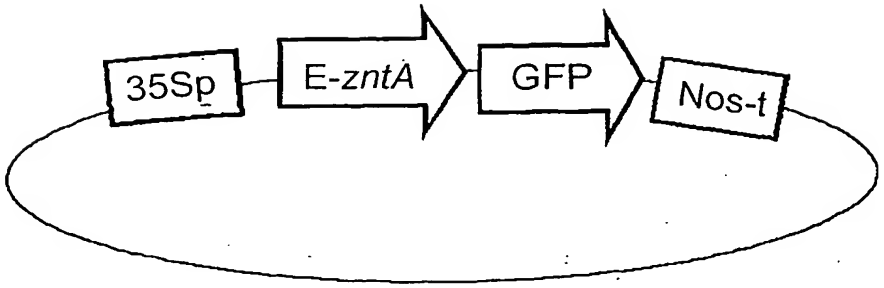


Fig. 2

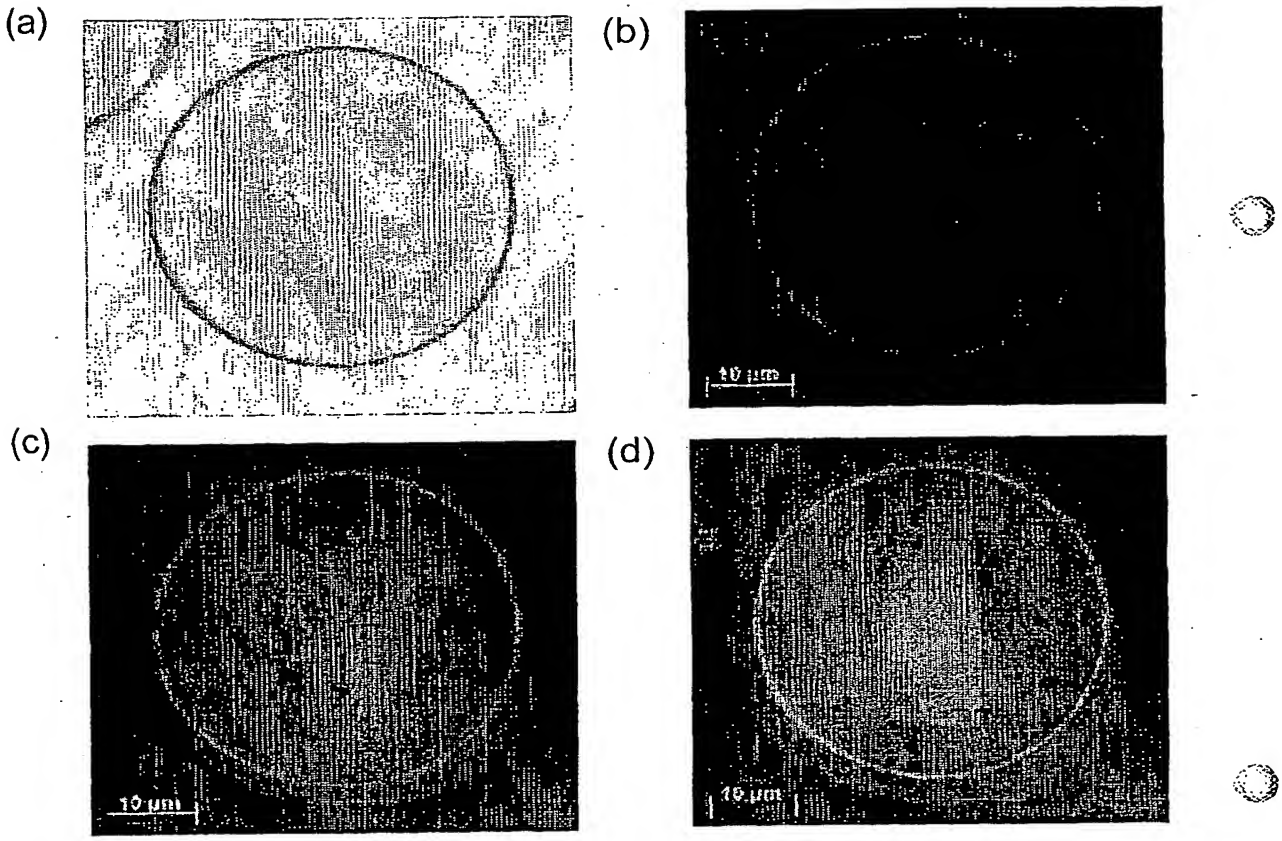
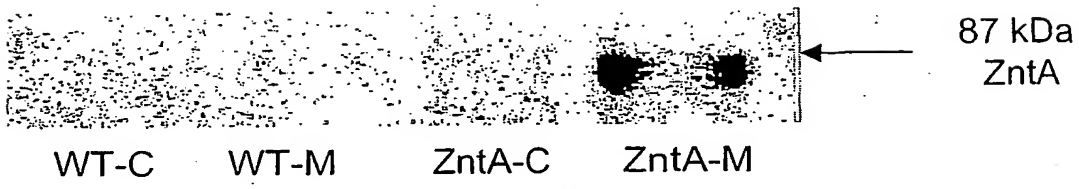
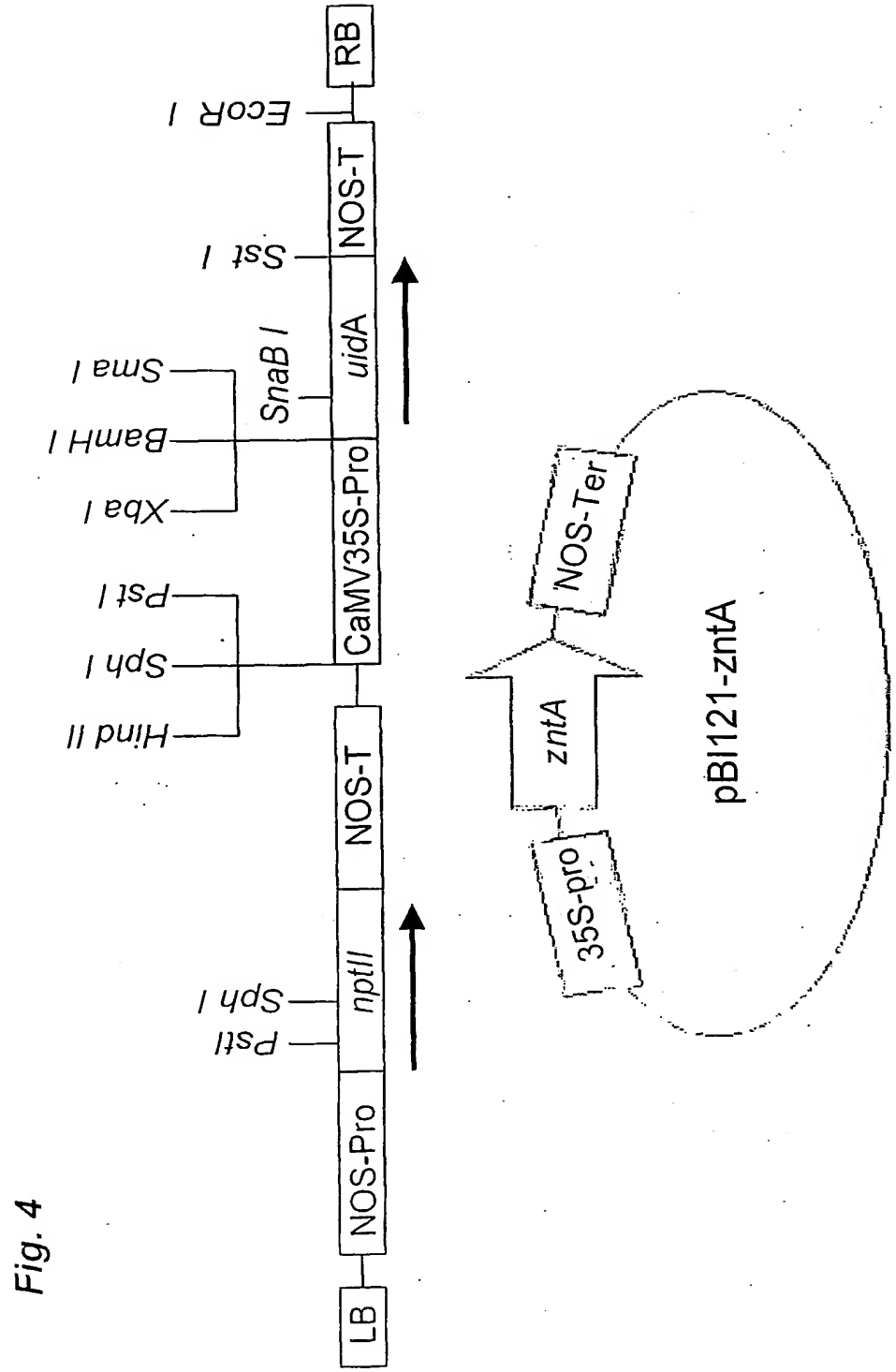


Fig. 3

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Hind II
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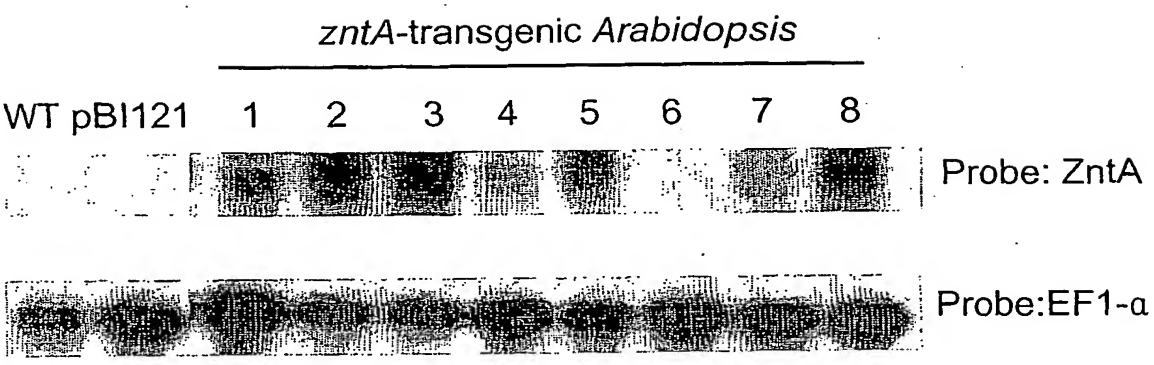
Fig. 4

Fig. 5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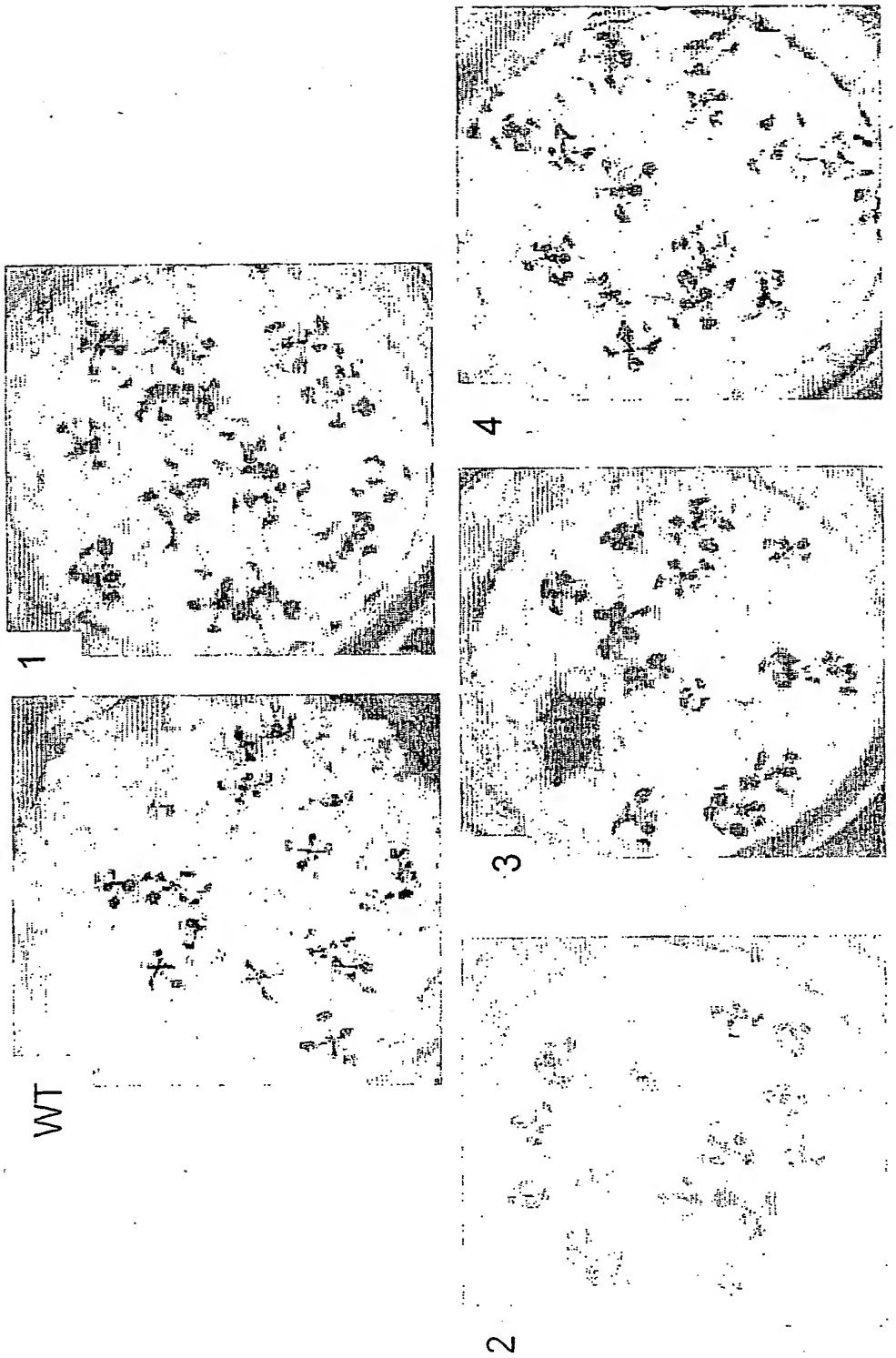

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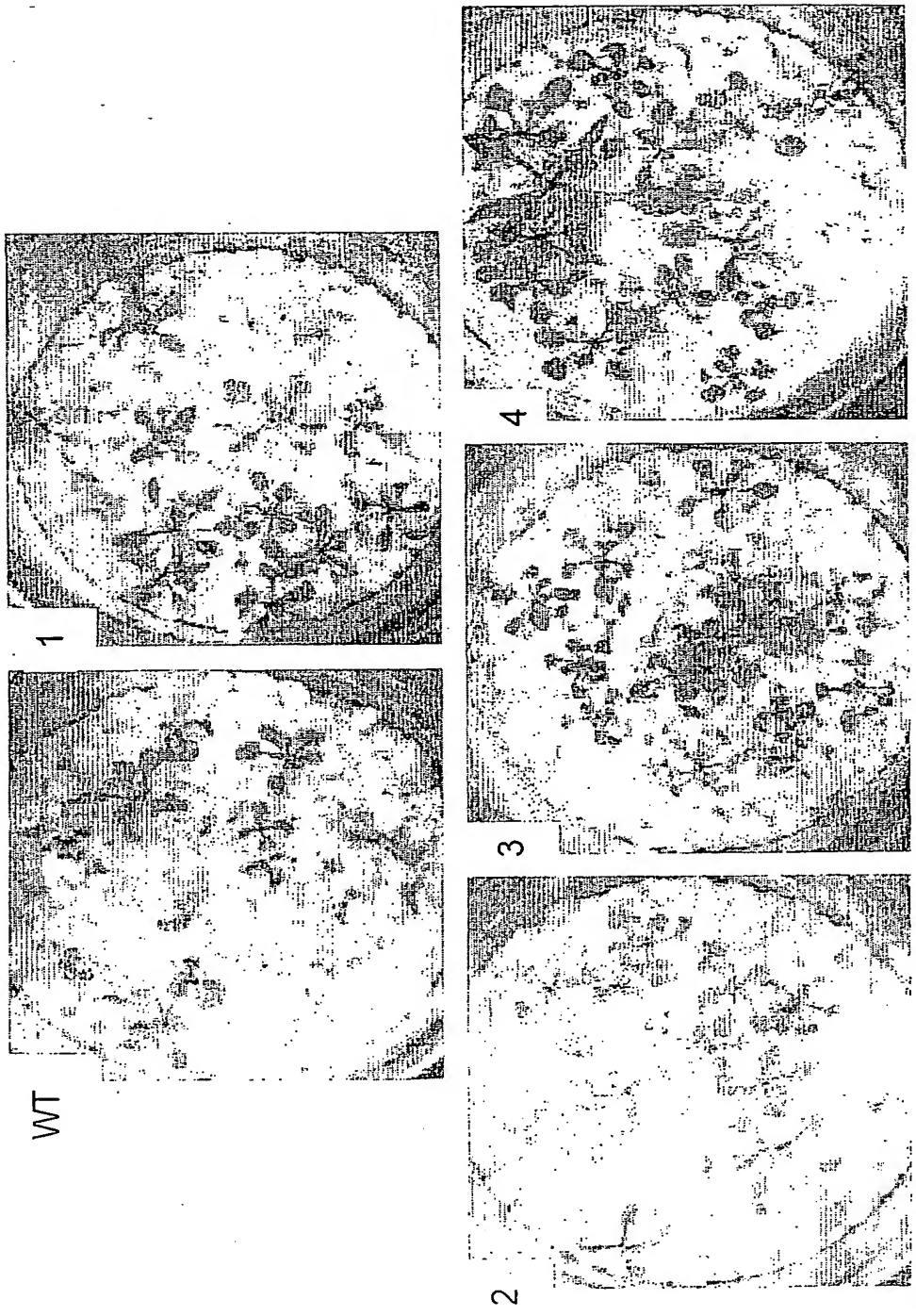

Fig. 7

Fig. 8a

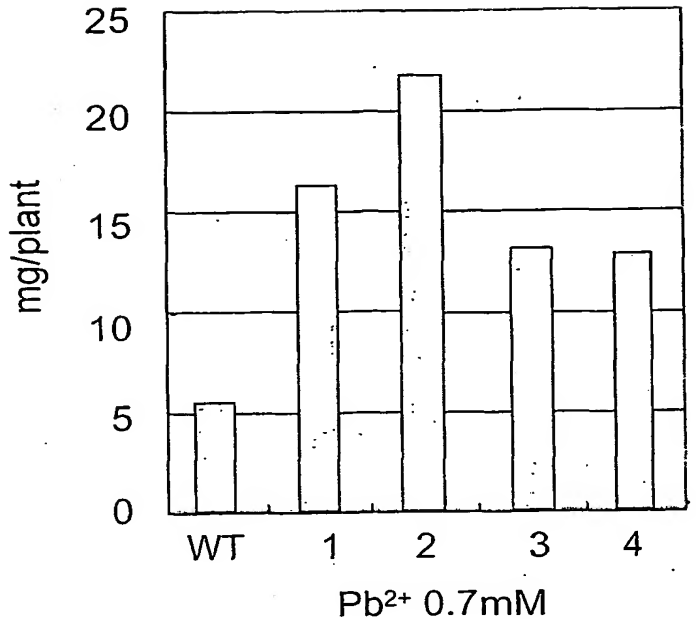
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Fig. 8b

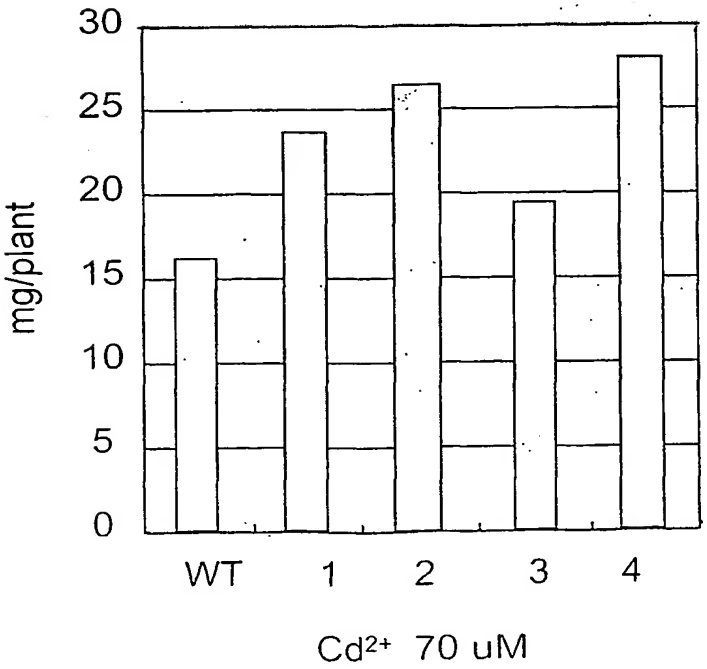
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Fig. 9a

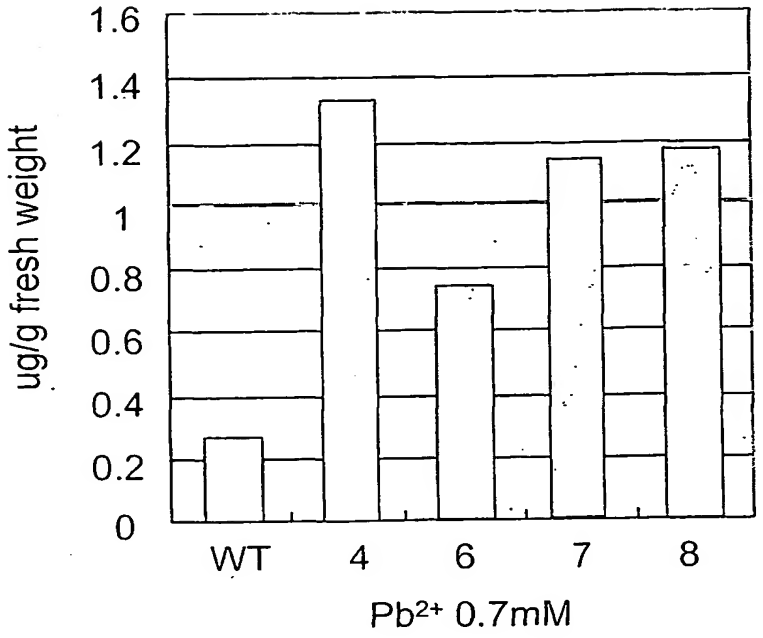
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Fig. 9b

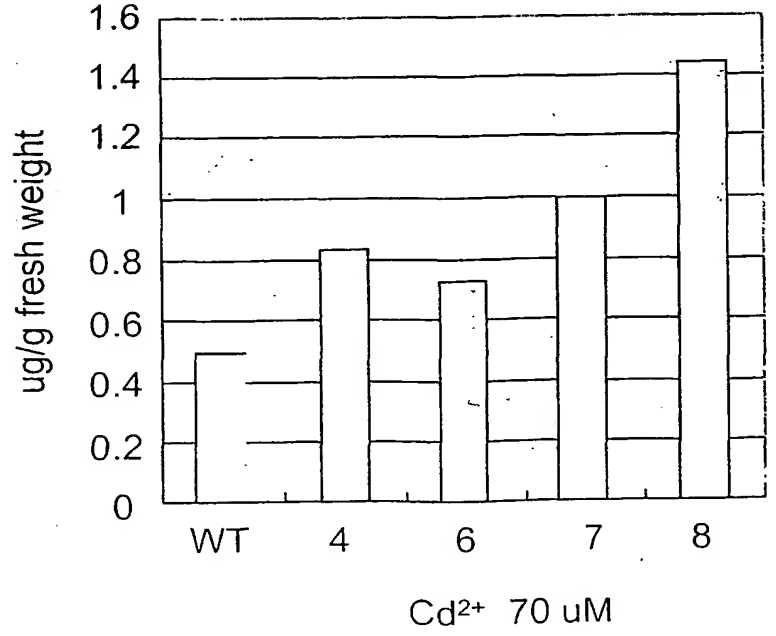
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Fig. 10a

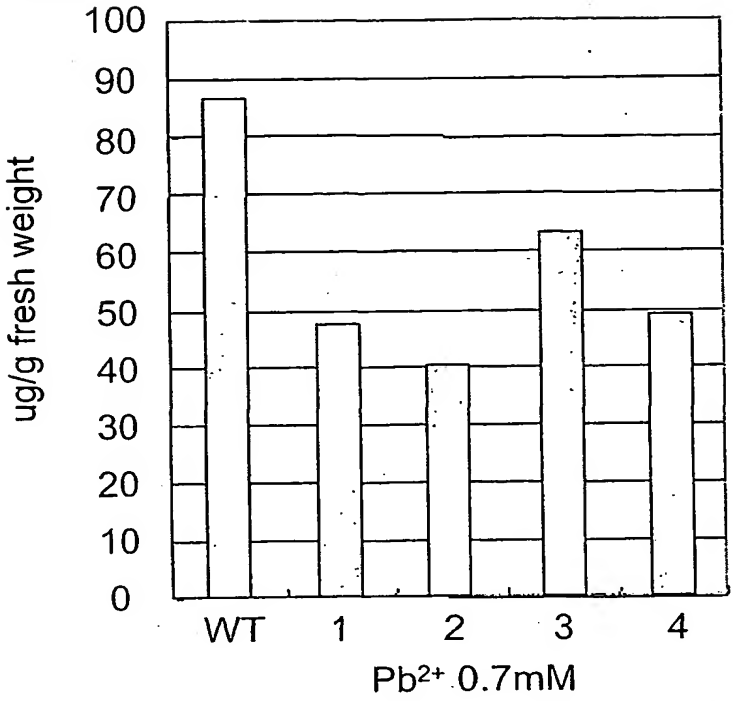
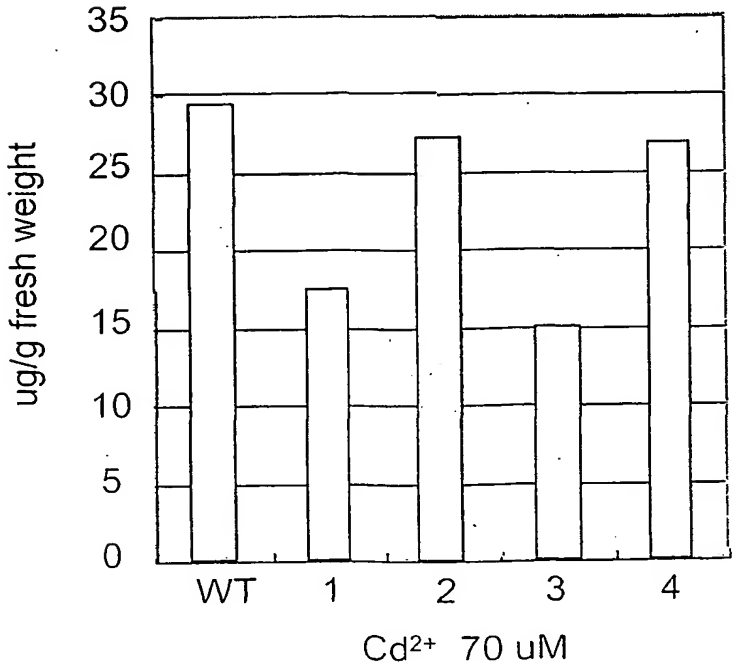


Fig. 10b



Sequence Listing

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Pohang University of Science and Technology

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Val Ala Phe Asp Lys Thr Gly Thr Leu Thr Val Gly Lys Pro Arg Val
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Thr Ala Ile His Pro Ala Thr Gly Ile Ser Glu Ser Glu Leu Leu Thr
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Leu Ala Ala Ala Val Glu Gln Gly Ala Thr His Pro Leu Ala Gln Ala
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Ile Val Arg Glu Ala Gln Val Ala Glu Leu Ala Ile Pro Thr Ala Glu
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Ser Gln Arg Ala Leu Val Gly Ser Gly Ile Glu Ala Gln Val Asn Gly
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Glu Arg Val Leu Ile Cys Ala Ala Gly Lys His Pro Ala Asp Ala Phe
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Thr Gly Leu Thr Asn Glu Leu Glu Ser Ala Gly Gln Thr Val Val Leu
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Val Val Arg Asn Asp Asp Val Leu Gly Val Ile Ala Leu Gln Asp Thr

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Leu Arg Ala Asp Ala Ala Thr Ala Ile Ser Glu Leu Asn Ala Leu Gly
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Ile Ala Gly Glu Leu Gly Leu Glu Phe Lys Ala Gly Leu Leu Pro Glu
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Asp Lys Val Lys Ala Val Thr Glu Leu Asn Gln His Ala Pro Leu Ala
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Met Val Gly Asp Gly Ile Asn Asp Ala Pro Ala Met Lys Ala Ala Ala
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Ile Gly Ile Ala Met Gly Ser Gly Thr Asp Val Ala Leu Glu Thr Ala
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Asp Ala Ala Leu Thr His Asn His Leu Arg Gly Leu Val Gln Met Ile
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Glu Leu Ala Arg Ala Thr His Ala Asn Ile Arg Gln Asn Ile Thr Ile
675 680 685

Ala Leu Gly Leu Lys Gly Ile Phe Leu Val Thr Thr Leu Leu Gly Met
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Val Thr Ala Asn Ala Leu Arg Leu Leu Arg Arg Arg
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<400> 6
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR 02/00605

CLASSIFICATION OF SUBJECT MATTER

IPC[7]: C12N 15/55, 15/31, 15/82, 5/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC[7]: C12N 15/55, 15/31, 15/82, 5/10

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and. where practicable. search terms used)

WPI, CAS, STN-registry, Medline

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 00/04760 A1 (THE TRUSTEES OF THE UNIVERSITY OF PENNSYLVANIA) 3 February 2000 (03.02.00) *page 41, lines 17,18; page 45, line 17 - page 46, line 19; claims 1,2,7-10,16,18,21,29.*	1,2,7-12,33-35
X,P	EP 1136558 A1 (VLAAMSE INSTELLING VOOR TECHNOLOGISCH ONDERZOEK) 26 September 2001 (26.09.01) *page 1, line 49 - page 2, line 44; claims 1-4.*	1,2,7-12,33-35
A	WO 99/61616 A2 (YEDA RESEARCH AND DEVELOPMENT COMPANY LTD., FLANDER INTERUNIVERSITY INSTITUTE FOR BIOTECHNOLOGY) 2 December 1999 (02.12.99) *page 1, lines 26-30; claims 1,6,11-13,15,19,21,26.*	1-3,7-12,33-35
A	WO 97/45000 A1 (TRUSTEES OF DARTHMOUTH COLLEGE) 4 December 1997 (04.12.97) *abstract; claims 30,36,46,55; figure 24.*	1-3,7-12,33-35

☒ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

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„O" document referring to an oral disclosure, use, exhibition or other means
„P" document published prior to the international filing date but later than the priority date claimed
„T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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„&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 June 2002 (12.06.2002)	20 June 2002 (20.06.2002)
Name and mailing adress of the ISA/AT Austrian Patent Office Kohlmarkt 8-10; A-1014 Vienna Facsimile No. 1/53424/535	Authorized officer MOSSER Telephone No. 1/53424/437

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR 02/00605

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	AXELSEN K.B. et al. Evolution of substrate specificities in the P-type ATPase superfamily. Journal of Molecular Evolution (1998), 46(1), pages 84-101. *the whole document.*	1,2,7-12,33-35

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/KR 02/00605-0

Patent document cited in search report			Publication date	Patent family member(s)			Publication date
EP	A1	1136558	26-09-2001	AU	A5	00142123	03-10-2001
				WO	A2	00170989	27-09-2001
				WO	A3	00170989	11-04-2002
WO	A	4760				none	
WO	A1	9745000	04-12-1997	AU	A1	11423/97	05-01-1998
				CA	AA	2187728	29-11-1997
				US	A	5846821	08-12-1998
				US	A	6162900	19-12-2000
WO	A2	9961616	02-12-1999	AU	A1	40562/99	13-12-1999
WO	A3	9961616	13-04-2000	IL	A0	124653	06-12-1998